

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 9/20, C11D 3/386	A1	(11) International Publication Number: WO 00/60063 (43) International Publication Date: 12 October 2000 (12.10.00)
(21) International Application Number: PCT/DK00/00156 (22) International Filing Date: 30 March 2000 (30.03.00) (30) Priority Data: PA 1999 00441 31 March 1999 (31.03.99) DK 60/128,933 13 April 1999 (13.04.99) US (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Enzyme Business Patents, Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): VIND, Jesper [DK/DK]; Bagsværdvej 115, DK-2800 Lyngby (DK). SVENDSEN, Allan [DK/DK]; Bakkeledet 28, DK-3460 Birkerød (DK). PATKAR, Shamkant, Anant [DK/DK]; Christoffers Allé 91, DK-2800 Lyngby (DK). ANDERSEN, Kim, Vilbør [DK/DK]; Tåsingevej 31 4.th, DK-2100 Copenhagen Ø (DK). HALKIER, Dorte, Aaby [DK/DK]; Hestkøvej 11E, DK-3460 Birkerød (DK). BOJSEN, Kirsten [DK/DK]; Bakkedal 5, DK-2900 Hellerup (DK).	(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: LIPASE VARIANT		
(57) Abstract		
<p>Certain variants of Lipolase (wild-type <i>Humicola lanuginosa</i> lipase) have a particularly good first-wash performance in a detergent solution. The variants should comprise one or more substitutions with positive amino acids near the N-terminal in the three-dimensional structure. The variants should further comprise a peptide addition at the C-terminal and/or should meet certain limitations on electrically charged amino acids at positions 90-101 and 210.</p>		

NZNA-0028707

19

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

LIPASE VARIANT

FIELD OF THE INVENTION

The present invention relates to lipase variants suited for use in detergent compositions. More particularly, the invention relates to variants of the wild-type
5 lipase from *Humicola lanuginosa* strain DSM 4109 showing a first-wash effect.

BACKGROUND OF THE INVENTION

For a number of years, lipases have been used as detergent enzymes to remove lipid or fatty stains from clothes and other textiles, particularly a lipase derived from *Humicola lanuginosa* (EP 258 068 and EP 305 216) sold under the
10 tradename Lipolase® (product of Novo Nordisk A/S).

WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and WO 97/07202 disclose variants of the *H. lanuginosa* lipase having improved properties for detergent purposes. Thus, WO 97/04079 discloses variants having a peptide addition (extension) at the N-terminal and/or the C-terminal. WO 97/07202 discloses
15 lipase variants with "first wash performance" which are capable of removing substantial amounts of lard from a lard stained swatch in a one-cycle wash.

There is an ever existing need for providing novel lipases with improved washing properties in a variety of commercial detergents. The present invention relates to such novel lipases.

20 SUMMARY OF THE INVENTION

The inventors have found that certain variants of Lipolase (wild-type *Humicola lanuginosa* lipase) have a particularly good first-wash performance in a detergent solution. The lipases may further provide additional benefits, such as whiteness maintenance and dingy cleanup.

25 The inventors found that the variants should comprise one or more substitutions with positive amino acids near the N-terminal in the three-dimensional structure. The variants should further comprise a peptide addition at the C-terminal and/or should meet certain limitations on electrically charged amino acids at positions 90-101 and 210.

30 Accordingly, the invention provides a lipase which is a polypeptide having an amino acid sequence which:

a) has at least 90 % identity with the wild-type lipase derived from *Humicola lanuginosa* strain DSM 4109;

b) compared to said wild-type lipase, comprises a substitution of an electrically neutral or negatively charged amino acid at the surface of the three-dimensional structure within 15 Å of E1 or Q249 with a positively charged amino acid; and

c) comprises a peptide addition at the C-terminal; and/or

d) meets the following limitations:

i) comprises a negative amino acid in position E210 of said wild-type lipase;

10 ii) comprises a negatively charged amino acid in the region corresponding to positions 90-101 of said wild-type lipase; and

iii) comprises a neutral or negative amino acid at a position corresponding to N94 of said wild-type lipase and/or has a negative or neutral net electric charge in the region corresponding to positions 90-101 of said wild-type lipase.

15 DETAILED DESCRIPTION OF THE INVENTION

Humicola lanuginosa lipase

The reference lipase used in this invention is the wild-type lipase derived from *Humicola lanuginosa* strain DSM 4109. It is described in EP 258 068 and EP 305 216 and has the amino acid sequence shown in positions 1-269 of SEQ ID NO: 20 2 of US 5,869,438. In this specification, the reference lipase is also referred to as Lipolase.

Substitution with positive amino acid

The lipase of the invention comprises one or more (e.g. 2-4, particularly two) substitutions of an electrically neutral or negatively charged amino acid near E1 or 25 Q249 with a positively charged amino acid, preferably R.

The substitution is at the surface of the three-dimensional structure within 15 Å of E1 or Q249, e.g. at any of positions 1-11, 90, 95, 169, 171-175, 192-211, 213-226, 228-258, 260-262.

The substitution may be within 10 Å of E1 or Q249, e.g. at any of positions 1-30 7, 10, 175, 195, 197-202, 204-206, 209, 215, 219-224, 230-239, 242-254.

The substitution may be within 15 Å of E1, e.g. at any of positions 1-11, 169, 171, 192-199, 217-225, 228-240, 243-247, 249, 261-262.

The substitution is most preferably within 10 Å of E1, e.g. at any of positions 1-7, 10, 219-224 and 230-239.

Thus, some preferred substitutions are S3R, S224R, P229R, T231R, N233R, D234R and T244R.

Peptide addition at C-terminal

The lipase may comprise a peptide addition attached to C-terminal L269.

5 The peptide addition improves the first-wash performance in a variety of detergents.

The peptide addition preferably consists of 1-5 amino acids, e.g. 2, 3 or 4 amino acids. The amino acids of the peptide addition will be numbered 270, 271, etc.

The peptide addition may consist of electrically neutral (e.g. hydrophobic) amino acids, e.g. PGL or PG. In an alternative embodiment, the lipase peptide
10 addition consists of neutral (e.g. hydrophobic) amino acids and the amino acid C, and the lipase comprises substitution of an amino acid with C at a suitable location so as to form a disulfide bridge with the C of the peptide addition. Examples are:

270C linked to G23C or T37C

271C linked to K24C, T37C, N26C or R81C

15 272C linked to D27C, T35C, E56C, T64C or R81C.

Amino acids at positions 90-101 and 210

The lipase of the invention preferably meets certain limitations on electrically charged amino acids at positions 90-101 and 210. Lipases meeting the charge limitations are particularly effective in a detergent with high content of anionic.

20 Thus, amino acid 210 may be negative. E210 may be unchanged or it may have the substitution E210D/C/Y, particularly E210D.

The lipase may comprise a negatively charged amino acid at any of positions 90-101 (particularly 94-101), e.g. at position D96 and/or E99.

Further, the lipase may comprise a neutral or negative amino acid at position
25 N94, i.e. N94(neutral or negative), e.g. N94N/D/E.

Also, the lipase may have a negative or neutral net electric charge in the region 90-101 (particularly 94-101), i.e. the number of negative amino acids is equal to or greater than the number of positive amino acids. Thus, the region may be unchanged from Lipolase, having two negative amino acids (D96 and E99) and one
30 positive (K98), and having a neutral amino acid at position 94 (N94), or the region may be modified by one or more substitutions.

Alternatively, two of the three amino acids N94, N96 and E99 may have a negative or unchanged electric charge. Thus, all three amino acids may be unchanged or may be changed by a conservative or negative substitution, i.e.
35 N94(neutral or negative), D(negative) and E99(negative). Examples are N94D/E and

D96E. Also, one of the three may be substituted so as to increase the electric charge, i.e. N94(positive), D96(neutral or positive) or E99 (neutral or positive). Examples are N94K/R, D96I/L/N/S/W or E99N/Q/K/R/H.

- 5 The substitution of a neutral with a negative amino acid (N94D/E), may improve the performance in an anionic detergent. The substitution of a neutral amino acid with a positive amino acid (N94K/R) may provide a variant lipase with good performance both in an anionic detergent and in an anionic/non-ionic detergent (a detergent with e.g. 40-70 % anionic out of total surfactant).

Amino acids at other positions

- 10 The inventors have found that a substitution Q249R/K/H may improve the performance both in anionic and in anionic/non-ionic detergent, and that a substitution of R209 with a neutral or negative amino acid (e.g. R209P/S) may improve the performance in anionic detergent. The lipase may optionally comprise the substitution G91A.

- 15 The lipase may optionally comprise substitutions of one or more additional amino acids. Such substitutions may, e.g., be made according to principles known in the art, e.g. substitutions described in WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and WO 97/07202.

Combinations of substitutions

- 20 A lipase variant with good first-wash performance may be obtained by modifying Lipolase as follows. Substitutions in parentheses are optional.

T231R+ N233R
N94K+ D96L+ T231R+ N233R+ Q249R+ 270P+ 271G+ 272L
D96L+ T231R+ N233R
G91A+ E99K+ T231R+ N233R+ Q249R
(N33Q) +D96L +T231R +N233R +Q249R +270 PGL
R209P +T231R +N233R
(N33Q) +E99N +N101S +T231R +N233R +Q249R +270 PGL
K24C +(N33Q) +D96S +T231R +N233R +Q249R +270 PCL
(N33Q) +G91A +E99K +T231R +N233R +Q249R +270 PGL
E1A +(N33Q) +G91A +E99K +T231R +N233R +Q249R +270 PGL
(N33Q) +G91A +E99K +G255R +T231R +N233R +Q249R +270 PGL
(N33Q) +G91A +E99K +T231R +N233R +T244R +Q249R +270 PGL

G91A +E99K +T231R +N233R +Q249R
E87K +G91D +D96L +G225P +T231R +N233R +Q249R +N251D
G91A +E99K +T231R +N233R +Q249R +270AGVF
G91A +E99K +T189G +T231R +N233R +Q249R
D102G +T231R +N233R +Q249R
T231R +N233R +Q249R +270AGVF
R209P +T231R +N233R
N33Q +N94K +D96L +T231R +N233R +Q249R +270PGLPFKRV
N33Q +N94K +D96L +T231R +N233R +Q249R
N33Q +D96S +T231R +N233R +Q249R
N33Q +D96S +V228I + +T231R +N233R +Q249R
E1A +N33Q +G91A +E99K +T231R +N233R +Q249R +270PGLPFKRV
N33Q +S83T +E87K +G91A + E99K +T231R +N233R +Q249R +270PGLPFKRV
N33Q +G91A + E99K +T231R +N233R +Q249R +270PGLPFKRV
T231R +N233R +270CP
T231R +N233R +270RE
N33Q +E99N +N101S +T231R +N233R +Q249R +270PGLPFKRV
D62A +S83T + G91A +E99K +T231R +N233R +Q249R
E99N +N101S +T231R +N233R +Q249R
R84W +G91A +E99K +T231R +N233R +Q249R
G91A +E99K +T231R +N233R +Q249R +270SPG
G91A +E99K +T231R +N233R +Q249R +270VVVP
G91A +E99K +T231R +N233R +Q249R +270LLASSGRGGHR
G91A +E99K +T231R +N233R +Q249R +270VTT
G91A +E99K +T231R +N233R +Q249R +270VLQ
G91A +E99K +T231R +N233R +Q249R +270TST
G91A +E99K +T231R +N233R +Q249R +270LRI
V60G +D62E +G91A +E99K +T231R +N233R +Q249R
G91A +D96W +E99K +T231R +N233R +G263Q +L264A +I265T +G266S +T267A +L269N +270AGGFS

Nomenclature for amino acid modifications

The nomenclature used herein for defining mutations is essentially as described in WO 92/05249. Thus, T231R indicates a substitution of T in position 231

with R. PGL or 270P+ 271G+ 272L indicates the peptide addition PGL attached to the C-terminal (L269).

Amino acid grouping

In this specification, amino acids are classified as negatively charged, 5 positively charged or electrically neutral according to their electric charge at pH 10, which is typical of the detergent of the invention. Thus, negative amino acids are E, D, C (cysteine) and Y, particularly E and D. Positive amino acids are R, K and H, particularly R and K. Neutral amino acids are G, A, V, L, I, P, F, W, S, T, M, N, Q and C when forming part of a disulfide bridge. A substitution with another amino acid in 10 the same group (negative, positive or neutral) is termed a conservative substitution.

The neutral amino acids may be divided into hydrophobic (G, A, V, L, I, P, F, W and C as part of a disulfide bridge) and hydrophilic (S, T, M, N, Q).

Amino acid identity

The lipase variant of the of the invention has an amino acid identity of at 15 least 90 % (preferably more than 95 % or more than 98 %) with Lipolase.

The degree of identity may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, 20 S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

DNA sequence, Expression vector, Host cell, Production of lipase

The invention provides a DNA sequence encoding the lipase of the 25 invention, an expression vector harboring the DNA sequence, and a transformed host cell containing the DNA sequence or the expression vector. These may be obtained by methods known in the art.

The invention also provides a method of producing the lipase by culturing the transformed host cell under conditions conducive for the production of the lipase 30 and recovering the lipase from the resulting broth. The method may be practiced according to principles known in the art.

Detergent additive

According to the invention, the lipase may typically be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme. The additive may be prepared by methods known in the art.

DETERGENT COMPOSITION

The detergent compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The detergent composition of the invention comprises the lipase of the invention and a surfactant. Additionally, it may optionally comprise a builder, another enzyme, a suds suppresser, a softening agent, a dye-transfer inhibiting agent and other components conventionally used in detergents such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or non-encapsulated perfumes.

The detergent composition according to the invention can be in liquid, paste, gels, bars or granular forms. The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11, particularly 9-11. Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. from 550 to 950 g/l.

The lipase of the invention, or optionally another enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level from 0.01% to 0.2% of enzyme protein by weight of the composition.

The detergent composition of the invention may comprise the lipase in an amount corresponding to 10-50,000 LU per gram of detergent, preferably 20-5,000 LU/g, e.g. 100-1000 LU/g. The detergent may be dissolved in water to produce a wash liquor containing lipolytic enzyme in an amount corresponding to 25-15,000 LU per liter of wash liquor, particularly 100 - 5000 LU/l, e.g. 300-2000 LU/l. The amount

of lipase protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

More specifically, the lipase of the invention may be incorporated in the detergent compositions described in WO 97/04079, WO 97/07202, WO 97/41212,
5 PCT/DK WO 98/08939 and WO 97/43375.

Surfactant system

The surfactant system may comprise nonionic, anionic, cationic, ampholytic, and/or zwitterionic surfactants. As described above, the lipase variants of the invention are particularly suited for detergents comprising of a combination of
10 anionic and nonionic surfactant with 70-100 % by weight of anionic surfactant and 0-30 % by weight of nonionic, particularly 80-100 % of anionic surfactant and 0-20 % nonionic. As further described, some preferred lipases of the invention are also suited for detergents comprising 40-70 % anionic and 30-60 % non-ionic surfactant.

The surfactant is typically present at a level from 0.1% to 60% by weight,
15 e.g. 1% to 40%, particularly 10-40 %. preferably from about 3% to about 20% by weight. Some examples of surfactants are described below.

Anionic surfactants

Preferred anionic surfactants include alkyl sulfate, alkyl ethoxy sulfate, linear alkyl benzene sulfonate and mixtures of these.

20 The alkyl sulfate surfactants are water soluble salts or acids of the formula ROSO_3M wherein R preferably is a $\text{C}_{10}\text{-C}_{24}$ hydrocarbyl, preferably an alkyl or hydroxyalkyl having a $\text{C}_{10}\text{-C}_{20}$ alkyl component, more preferably a $\text{C}_{12}\text{-C}_{18}$ alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium.

25 Alkylbenzene sulfonates are suitable, especially linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group preferably contains from 10 to 18 carbon atoms.

Suitable anionic surfactants include alkyl alkoxyated sulfates which are water soluble salts or acids of the formula $\text{RO(A)}_m\text{SO}_3\text{M}$ wherein R is an
30 unsubstituted $\text{C}_{10}\text{-C}_{24}$ alkyl or hydroxyalkyl group having a $\text{C}_{10}\text{-C}_{24}$ alkyl component, preferably a $\text{C}_{12}\text{-C}_{20}$ alkyl or hydroxyalkyl, more preferably $\text{C}_{12}\text{-C}_{18}$ alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more preferably between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium,
35 lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl

ethoxylated sulfates as well as alkyl propoxylated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl-, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and dimethyl piperdinium cations and those derived from alkylamines
5 such as ethylamine, diethylamine, triethylamine, mixtures thereof, and the like.

Other anionic surfactants include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C₈-C₂₂ primary or secondary alkanesulfonates, C₈-C₂₄ olefinsulfonates, sulfonated polycarboxylic acids prepared by sulfonation of the
10 pyrolyzed product of alkaline earth metal citrates.

Nonionic surfactant

The surfactant may comprise polyalkylene oxide (e.g. polyethylene oxide) condensates of alkyl phenols. The alkyl group may contain from about 6 to about 14 carbon atoms, in a straight chain or branched-chain. The ethylene oxide may be
15 present in an amount equal to from about 2 to about 25 moles per mole of alkyl phenol.

The surfactant may also comprise condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide. The alkyl chain of the aliphatic alcohol can either be straight or branched, and generally
20 contains from about 8 to about 22 carbon atoms.

Further, the nonionic surfactant may comprise polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide, alkylpolysaccharides, and mixtures hereof. Most preferred are C₈-C₁₄ alkyl phenol
25 ethoxylates having from 3 to 15 ethoxy groups and C₈-C₁₈ alcohol ethoxylates (preferably C₁₀ avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Preferred nonionic surfactants are alcohol ethoxylate, alcohol phenol ethoxylate, polyhydroxy fatty acid amide, alkyl polyglucoside and mixtures of these.

EXAMPLE

30 First-wash performance at various dosages in anionic detergent

The following 5 variants according to the invention were prepared and tested with 3 types of soiled swatches, and the parent lipase was included for comparison:

T231R +N233R.
G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS
R209P +T231R +N233R
N33Q +D96S +T231R +N233R +Q249R
E99N +N101S +T231R +N233R +Q249R

A commercial US detergent with a high content of anionic surfactant was heated to inactivate the enzymes already present, and was used at 1.4 g/l in a Tergot-o-meterTM laboratory washing machine. The water hardness was 6°dH
 5 (Ca:Mg 2:1), and the washing conditions were 1 cycle at 30°C for 12 minutes, followed by over-night drying on filter paper. The lipase variant was added at dosages of 6400 and 12800 LU/l. Double determinations were made in two separate washes (same TOM).

The following three types of textile swatches tested were: Lard/Sudan Red
 10 on cotton Style 400, freshly prepared; wfk 20-LS Lipstick on polyester/cotton; and wfk 10-LS lipstick on cotton. The soil removal was evaluated by measuring the remission at 460 nm after the first washing cycle, and the results were expressed as ΔR by subtracting the remission of a blank without enzyme.

The results were that each variant at each dosage showed an improved first-
 15 wash performance on each type of soiled swatch (ΔR of 5 to 13), whereas the parent enzyme had essentially no effect ($\Delta R = 0 \pm 2$) under the same conditions.

CLAIMS

1. A lipase which is a polypeptide having an amino acid sequence which:
 - a) has at least 90 % identity with the wild-type lipase derived from *Humicola lanuginosa* strain DSM 4109;
 - 5 b) compared to said wild-type lipase, comprises a substitution of an electrically neutral or negatively charged amino acid at the surface of the three-dimensional structure within 15 Å of E1 or Q249 with a positively charged amino acid; and
 - c) comprises a peptide addition at the C-terminal; and/or
 - 10 d) meets the following limitations:
 - i) comprises a negative amino acid in position E210 of said wild-type lipase;
 - ii) comprises a negatively charged amino acid in the region corresponding to positions 90-101 of said wild-type lipase; and
 - 15 iii) comprises a neutral or negative amino acid at a position corresponding to N94 of said wild-type lipase and/or has a negative or neutral net electric charge in the region corresponding to positions 90-101 of said wild-type lipase.
2. The lipase of claim 1 wherein said substitution is within 10 Å of E1 or Q249.
- 20 3. The lipase of claim 1 or 2 wherein said substitution is within 15 Å (preferably 10 Å) of E1.
4. The lipase of any preceding claim which comprises 2-4 (preferably two) of said substitutions.
5. The lipase of any preceding claim which comprises a substitution of an
25 electrically neutral or negatively charged amino acid within 10 Å of E1 with R.
6. The lipase of any preceding claim which comprises a substitution of amino acid S3, S224, P229, T231, N233, D234 or T244 with a positively charged amino acid, preferably R.

7. The lipase of any preceding claim wherein the peptide addition consists of 1-5 amino acids.
8. The lipase of any preceding claim wherein the peptide addition consists of electrically neutral (preferably hydrophobic) amino acids, and is most preferably PGL
5 or PG.
9. The lipase of any of claims 1-7 wherein the peptide addition consists of neutral (preferably hydrophobic) amino acids and C, and the lipase further comprises substitution of an amino acid with C so as to form a disulfide bridge with the C of the peptide addition.
- 10 10. The lipase of any preceding claim which comprises amino acids with negative or unchanged electric charge in at least two of positions N94, D96 and E99 of said wild-type lipase.
11. The lipase of any preceding claim which comprises a substitution G91A, N94D/E/K/R, D96E/I/L/N/S/W, E99N/Q/K/R/H, N101S, R209P/S or Q249R/K/H.
- 15 12. The lipase of any preceding claim which comprises one of the following sets of substitutions, optionally combined with Q249R/K/H and/or K98X:
- a) G91G/A/S/T + N94(neutral or negative) + D96D/E/C/Y + E99E/D/C/Y,
 - b) G91G/A/S/T + N94(neutral or negative) + D96D/E/C/Y + E99N + N101S,
 - 20 c) G91G/A/S/T + N94R/K/H + D96D/E/C/Y + E99E/D/C/Y,
 - d) G91G/A/S/T + N94(neutral or negative) + D96(neutral or positive) + E99E/D/C/Y, or
 - e) G91G/A/S/T + N94(neutral or negative) + D96D/E/C/Y + E99(neutral or positive).
- 25 13. The lipase of any of claims 1-11 which comprises one of the following sets of substitutions, optionally combined with R209(neutral or negative):
- a) E99R/K/H + Q249R/K/H,
 - b) N94R/K/H + Q249R/K/H, or
 - c) D96(neutral or positive) + Q249R/K/H.

14. A detergent composition comprising a surfactant and the lipase of any of claims 1-13.
15. The detergent composition of the preceding claim wherein the surfactant comprises anionic surfactant in an amount of more than 70 % by weight of the total
5 surfactant.
16. The detergent composition of claim 14, wherein the surfactant comprises anionic surfactant in an amount of 40-70 % by weight and nonionic surfactant in an amount of 30-60 % by weight of the total surfactant, and the lipase is the lipase of claim 8 or 9.
- 10 17. The detergent composition of any of claims 14-16 which comprises 10-40 % by weight surfactant, preferably comprises 40-70 % builder, and preferably has a pH of 9-11 when dissolved in water at 0.5-5 g/l.
18. A DNA sequence encoding the lipase of any of claims 1-13.
19. An expression vector harboring the DNA sequence of the preceding claim.
- 15 20. A transformed host cell containing the DNA sequence of claim 18 or the expression vector of claim 19.
21. A method of producing the lipase of any of claims 1-13 which method comprises culturing the transformed host cell of claim 20 under conditions conducive for the production of the lipase and recovering the lipase from the resulting broth.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 00/00156

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/20, C11D 3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9707202 A1 (NOVO NORDISK A/S), 27 February 1997 (27.02.97), See especially page 21, lines 23-32; page 31, lines 22-26; page 34, line 26 - page 39, line 20; claim 12 --	1-21
P,X	WO 9942566 A1 (NOVO NORDISK A/S), 26 August 1999 (26.08.99), See especially abstract; page 1, lines 4-6; page 2, line 3 - page 5, line 22 --	1-21
A	WO 9522615 A1 (NOVO NORDISK A/S), 24 August 1995 (24.08.95) -----	1-21

☐ Further documents are listed in the continuation of Box C.
 ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

1 August 2000

Date of mailing of the international search report

04 -08- 2000

Name and mailing address of the ISA/

Swedish Patent Office

Box 5055, S-102 42 STOCKHOLM

Facsimile No. +46 8 666 02 86

Authorized officer

Henrik Nilsson/Els

Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00156

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5
because they relate to subject matter not required to be searched by this Authority, namely:
See next page
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

NZNA-0028723

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 00/00156

Claims 1-5 relate to a lipase in which certain amino acid substitutions have been made "at the surface of the three-dimensional structure within 15 Å of E1 or Q249...". The use of the parameter relating to the relative distances in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT, since the 3D-structure and hence the intended positions are not derivable only from a given distance. It is therefore impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a complete search impossible. Consequently, the search has been restricted to the substitutions disclosed in the examples and in the independent claims.

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/12/99

International application No.

PCT/DK 00/00156

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9707202 A1	27/02/97	AU 6655196 A CN 1192780 A EP 0851913 A JP 11510699 T AU 6414096 A AU 6414196 A CN 1193346 A EP 0839186 A WO 9704078 A WO 9704079 A	12/03/97 09/09/98 08/07/98 21/09/99 18/02/97 18/02/97 16/09/98 06/05/98 06/02/97 06/02/97
WO 9942566 A1	26/08/99	NONE	
WO 9522615 A1	24/08/95	AU 1806795 A BR 9506861 A CA 2183431 A CN 1147836 A EP 0746618 A FI 963266 A JP 9509058 T US 5869438 A US 5892013 A US 5976855 A	04/09/95 23/09/97 24/08/95 16/04/97 11/12/96 21/08/96 16/09/97 09/02/99 06/04/99 02/11/99

Form PCT/ISA/210 (patent family annex) (July 1992)

NZNA-0028725